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THE BIALAPHOS RESISTANCE GENE (*bar*) PLAYS A ROLE IN  
BOTH SELF-DEFENSE AND BIALAPHOS BIOSYNTHESIS  
IN *STREPTOMYCES HYGROSCOPICUS*

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We inactivated the bialaphos (BA) resistance gene (*bar*) of a BA producer, *Streptomyces hygroscopicus*, by the gene replacement technique. The resulting BA-sensitive mutant ( $\text{Bar}^-$ ) was able to produce little BA but considerable amount of an intermediate demethylphosphinothricin (DMPT). The  $\text{Bar}^-$  mutant was still able to convert the *N*-acetyl derivative (AcDMPT) of DMPT to BA. Introduction of normal *bar* containing plasmid restored both BA resistance and BA biosynthesis to levels as high as the parental BA producer. By contrast, introducing a multi copy glutamine synthetase gene (*glnA*) into the  $\text{Bar}^-$  mutant restored BA resistance but not BA production. Thus, the *bar* gene plays a crucial role in both self-defense and a step of BA biosynthesis in the BA-producing *S. hygroscopicus*.

The bialaphos (BA) (L-phosphinothricyl-L-alanyl-L-alanine) is a herbicidal secondary metabolite which is produced by *Streptomyces hygroscopicus* or *Streptomyces viridochromogenes*<sup>1-3)</sup>. Its *N*-terminal residue, phosphinothricin (PT), is an inhibitor of glutamine synthetase (GS)<sup>3,4)</sup>. BA itself does not inhibit GS but it is decomposed to PT in the cell and inhibits GS. Although *S. hygroscopicus* GS is also inhibited by PT (S. IMAI; unpublished), the organism has an detoxication enzyme (phosphinothricin acetyltransferase: PAT)<sup>5)</sup> so as to be resistant to PT and BA.

The structural gene (*bar*) of PAT that was cloned by THOMPSON *et al.*<sup>5)</sup> conferred BA-resistance to *Streptomyces lividans*, *Escherichia coli* and plants<sup>6)</sup>. It is known that the *bar* gene is involved in the BA biosynthetic gene cluster<sup>7)</sup>. Transcription of the *bar* gene as well as the other BA biosynthetic genes is regulated by a regulatory gene *brpA* which is located in the down stream of the cluster<sup>8)</sup>.

Thus it seemed likely that PAT performed a crucial role in both self-resistance and BA biosynthesis of BA-producing *S. hygroscopicus*. In order to obtain a conclusive evidence for this, we generated a  $\text{Bar}^-$  mutant, in which the *bar* gene was inactivated specifically by a gene replacement technique<sup>9,10)</sup> and examined its ability concerning BA resistance and BA biosynthesis. In addition, effect of normal *bar* and *glnA* genes on the  $\text{Bar}^-$  mutant was examined.

## Materials and Methods

### Plasmids and Bacterial Strains

*S. lividans* 1326 and plasmid pIJ702<sup>11)</sup> were obtained from John Innes Culture Collection. The bialaphos producer *S. hygroscopicus* HP5-29 was obtained from the Meiji Seika Culture Collection. A normal *bar* plasmid pBG3<sup>9)</sup> was supplied by THOMPSON and used for transformation of  $\text{Bar}^-$  mutant.

The transformant was designated Bar<sup>-</sup>/pBG3. Plasmid pSF6105, the complex plasmid containing the *glnA* gene<sup>12)</sup> from *Streptomyces coelicolor* and pIJ610 were supplied by S. FISHER. The *S. coelicolor glnA* gene (2.2 kb *Sac* I fragment) was subcloned into the *Sac* I site of pIJ702 from the plasmid pSF6105<sup>12)</sup>. The subcloned plasmid named pGSC1 (Fig. 1) was used for transformation of the Bar<sup>-</sup> mutant. The transformant was designated Bar<sup>-</sup>/pGSC1.

#### Media and Conditions for BA Fermentation

Media and conditions for BA production and BA assay were described before<sup>7,13)</sup>. Salvage synthesis of BA from *N*-acetyldemethylphosphinothricin (AcDMPT) was carried out as follows. Strains were precultured in 10 ml of S1 medium at 28°C for 1 day and 1 ml of the seed was transferred to 30 ml of the production medium<sup>7,13)</sup>. 60 mg of AcDMPT was added at 2, 4 and 6 days (total amount, 180 mg) and the culture was further cultivated for 2 or 3 days.

#### Preparation of BA Intermediates

Demethylphosphinothricin (DMPT), BA, PT and AcDMPT were prepared as described by IMAI *et al.*<sup>14,15)</sup> and SETO *et al.*<sup>13)</sup>.

#### Protoplast Transformation and DNA Manipulation

Plasmids and genomic DNA were prepared by previously described techniques<sup>7)</sup>. The preparation and transformation of protoplasts was described before<sup>7)</sup>. Restriction enzymes, T4 DNA ligase (Takara Shuzo Co., Ltd.), calf intestinal alkaline phosphatase and S1 DNA nuclease were used according to supplier's recommendations. Agarose gel electrophoresis of DNA was carried out according to HOPWOOD *et al.*<sup>16)</sup>.

#### Assay of BA and Intermediates

To extract the intracellular substances, culture broth was diluted with water, heated for 5 minutes in boiling water and centrifuged. The resulting supernatant was used for the assay. BA, PT, demethylbialaphos (DMBA), and DMPT were analyzed by an amino acid analyzer. *N*-Acetylbialaphos (AcBA), *N*-acetylphosphinothricin (AcPT), *N*-acetyldemethylbialaphos (AcDMBA), and AcDMPT were analyzed by HPLC. HPLC was carried out by using the anion exchanger ASAHI PACK ES-502N (3.2 i.d. × 150 mm) (Asahi Chemical Industry Co., Ltd.), and 70 mM NH<sub>4</sub>Cl as the moving phase (flow rate was 1 ml/minute at 45°C). Substances were monitored by the UV detector (210 nm). A bioassay was also carried out for quantitative analysis of BA production in the agar medium as follows. Agar plugs were inoculated with BA producers or non producers and incubated for 5~7 days at 28°C. The plugs were placed on the assay agar medium seeded with *Bacillus subtilis* ATCC 6633. BA production was determined by the diameter of the zone of growth inhibition.

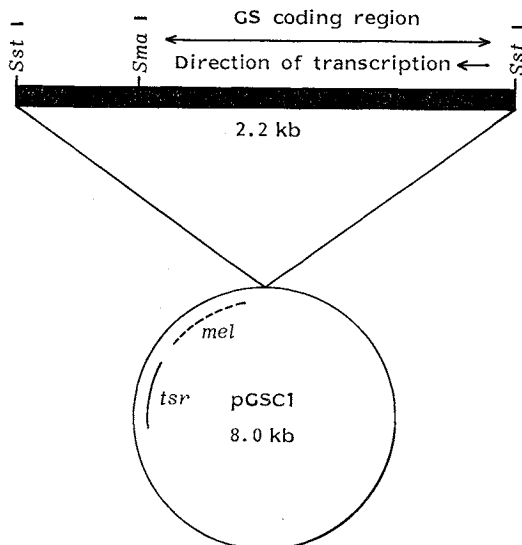
#### Measurement of the Resistance of Strains to BA, DMPT and PT

Strains were streaked on the minimal medium supplemented with various concentration of BA, DMPT or PT and were incubated at 28°C for 3 days.

#### Preparation of Cell Free Extracts and Analysis of Intracellular Protein

Cell free extracts were prepared as follows. Mycelium collected by centrifugation was washed with 50 mM Tris buffer (pH 7.5) containing 2 mM β-mercaptoethanol and resuspended in the same buffer. The mycelium was then disrupted by sonication and centrifuged (17,000 × *g*, for 15 minutes,

Fig. 1. Restriction map of pGSC1.



Circle line refers to pIJ702. GS: Glutamine synthetase, *tsr*: thiostrepton-resistance gene, *mel*: tylosinase gene (melanin formation).

at 4°C). Intracellular protein was analyzed by SDS-polyacrylamide gel electrophoresis according to LAEMMLI<sup>17)</sup>. Samples were denatured in a boiling water bath for 5 minutes in the presence of 1% SDS and 10% 2-mercaptoethanol before loading onto a 12.5%-polyacrylamide gel. SDS-PAGE Standard (Bio-Rad) was used as protein size markers.

#### Southern Hybridization

One  $\mu$ g of target DNAs digested with restriction enzymes were loaded onto 0.8% agarose gel. After electrophoresis, DNA in agarose gels was transferred onto a nylon membrane (Hybond-N, Amersham, Ltd.) by the method of SOUTHERN<sup>18)</sup> and the suppliers recommendation. 25 ng of DNA (pMSB307) was labeled by use of a Nick Translation Kit (Amersham, Ltd.) and used as the probe. Specific activity of the probe was 800  $\mu$ Ci/ $\mu$ g DNA. Prehybridization and hybridization were done as described by HOPWOOD *et al.*<sup>19)</sup>.

#### PAT Assay

PAT was assayed by the method of THOMPSON *et al.*<sup>5)</sup>.

#### Assay of Glutamine Synthetase

Formation of  $\gamma$ -glutamylhydroxamate from glutamate, hydroxylamine and ATP was assayed at pH 7.2 to estimate GS activity as described by BENDER *et al.*<sup>19)</sup>. One unit of GS activity is defined as the amount of enzyme producing 1  $\mu$ mol of glutamylhydroxamate per minute.

#### The Methods of Site Specific Inactivation

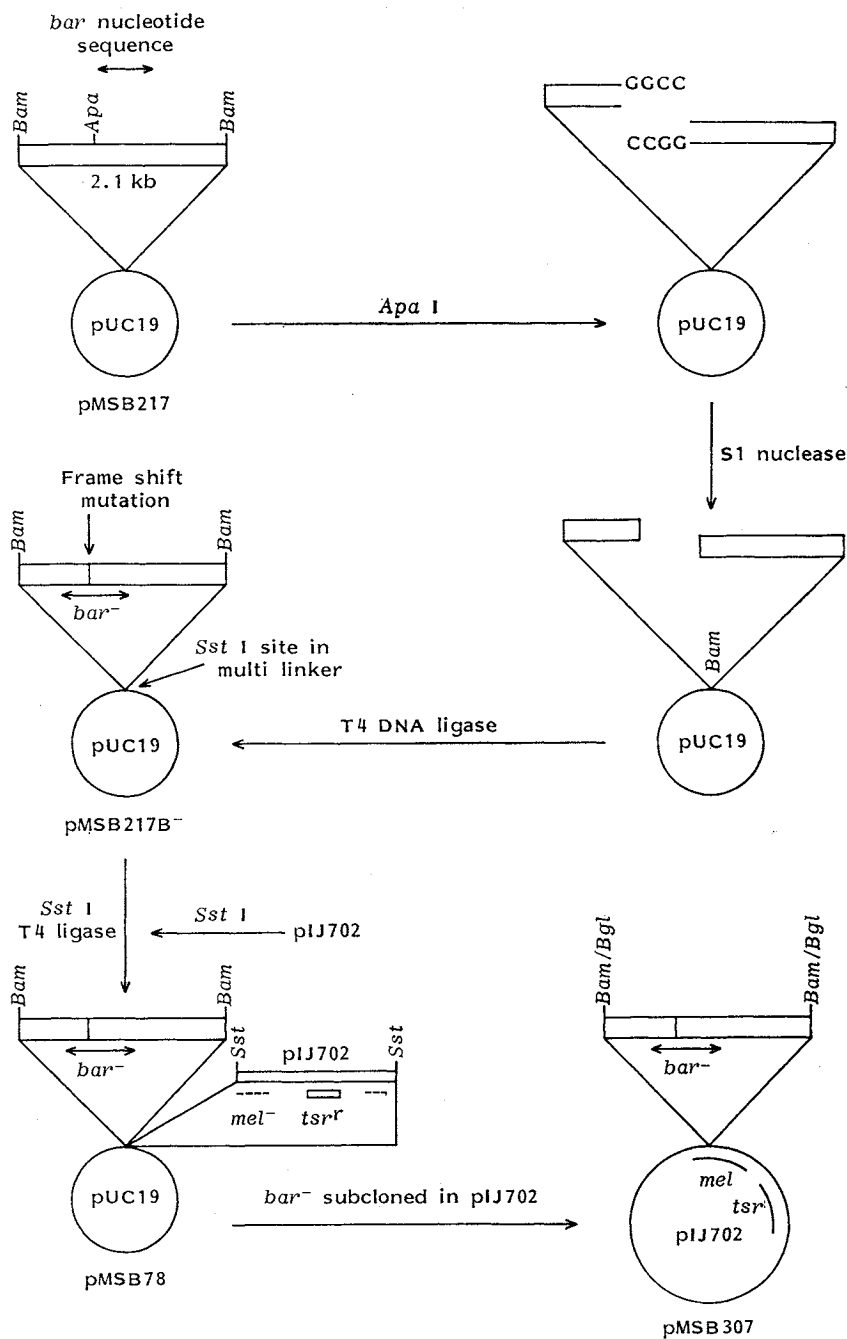
In principle, the method of KIEL *et al.*<sup>10)</sup> was used for the site specific inactivation of *bar* gene and the details were almost the same as that of ANZAI *et al.*<sup>9)</sup>.

### **Results**

#### **Construction of the *bar*<sup>-</sup> Gene and Derivation of a *Bar*<sup>-</sup> Mutant**

The *bar*<sup>-</sup> plasmid (pMSB307) was constructed by introducing a frame shift mutation into the *bar* region of pMSB217.

Fig. 2. shows the construction scheme of pMSB307. Plasmid pMSB217, consisting of the *bar* gene and an *E. coli* vector plasmid pUC19, was cleaved with the restriction enzyme *Apa* I of which unique site is located in the *bar* nucleotide sequence<sup>5)</sup>. The resultant cohesive ends were eliminated by S1 DNA nuclease to introduce a frame shift mutation into the *bar* gene. The resulting DNA was re-ligated with T4 DNA ligase and then digested with *Sst* I, *Apa* I to clone the 4.8-kb DNA fragment containing the inactivated *bar* gene (*bar*<sup>-</sup>) and pUC19 into *Streptomyces* vector plasmid pIJ702. The constructed plasmid (pMSB217B<sup>-</sup>) was isolated from an electrophoresed agarose gel and linked to pIJ702 cleaved with *Sst* I. *E. coli-Streptomyces* shuttle plasmid (pMSB78) containing the *bar*<sup>-</sup> gene thus constructed was introduced into *S. lividans* and transformants (thiostrepton-resistant, melanin formation-negative) were isolated. The transformants were replicated on the minimum medium supplemented with 100  $\mu$ g/ml BA to confirm that the transformant had BA-sensitive phenotype. The plasmid pMSB78 was re-extracted from the transformant and confirmed for the lack of the *Apa* I site in the *bar*<sup>-</sup> region by *Apa* I digestion. The *bar*<sup>-</sup> gene (2.1 kb *Bam*H I fragment) from pMSB78 was then subcloned into the *Bgl* II site of pIJ702. The resultant plasmid pMSB307 was then introduced into BA high producer *S. hygroscopicus* HP5-29. *S. hygroscopicus* HP5-29 harboring the *bar*<sup>-</sup> plasmid (pMSB307) generated mutants with *bar*<sup>-</sup> genotype at a high frequency during the multiplication. Twenty three out of one hundred transformants examined showed a significantly low (<1/100) BA production. One of the low producing transformants was cultured in a medium without thiostrepton, protoplasted and regenerated, One third of the regenerated colonies were low BA producers. Among

Fig. 2. Construction of the *bar*<sup>-</sup> plasmid.

*Bam*: *Bam*H I, *Sst*: *Sst* I, *Bgl*: *Bgl* II, *Apa*: *Apa* I.

these low producers, thiostrepton-sensitive (Thio<sup>s</sup>) strains lacking the plasmid were selected and their *bar*<sup>-</sup> genotype was confirmed by Southern hybridization as shown in Fig. 3. Total DNAs of the *Bar*<sup>-</sup> mutant and the parent strain (HP5-29) were subjected to hybridization with <sup>32</sup>P-labeled pMSB307 containing the *bar*<sup>-</sup> gene after digestion with *Bam*H I which was expected to provide a 2.1-kb DNA

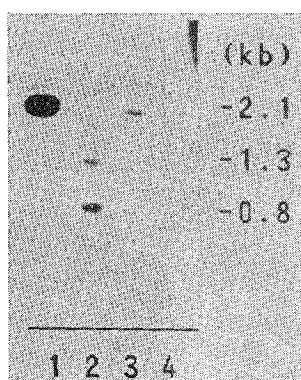
Table 1. Phenotypic properties of Bar<sup>-</sup> mutant.

Strain	Productivity <sup>a</sup> (%)		Resistance (μg/ml)		
	BA	DMPT	BA	DMPT	PT
Parent (HP5-29)	100.0	ND	10,000	>10,000	>10,000
Bar <sup>-</sup>	0.6	24.0	<10	100	<10
Bar <sup>-</sup> /pBG3 ( <i>bar</i> plasmid)	94.0	ND	10,000	>10,000	>10,000
Bar <sup>-</sup> /pGSC1 ( <i>glnA</i> plasmid)	0.6	20.0	10,000	>10,000	>10,000

<sup>a</sup> The productivity that was assayed in liquid cultures was expressed relative to the parent strain.

BA: Bialaphos, DMPT: demethylphosphinothricin, PT: phosphinothricin, ND: not detected.

Fig. 3. Southern hybridization of the chromosomal DNA.



The <sup>32</sup>P-labeled pMSB307 was hybridized to HP5-29 (lanes 1 and 2) and Bar<sup>-</sup> (lanes 3 and 4) chromosomal DNAs digested with *Bam*HI (lanes 1 and 3) and both *Bam*HI and *Apa*I (lanes 2 and 4).

fragment hybridizing to the probe. As expected, both DNA digests provided the same size positive band. In contrast, double digestion with *Bam*HI and *Apa*I gave different size positive bands. 2.1 kb fragment hybridizing to the probe was again detected in the Bar<sup>-</sup> mutant DNA and 0.8 kb were detected in the parent DNA digest.

These results indicate the lack of the *Apa*I site in the *bar*<sup>-</sup> gene.

#### Characterization of the Bar<sup>-</sup> Mutant

The Bar<sup>-</sup> mutant was examined for BA resistance, BA production and PAT activity as shown in Tables 1 and 2. The resistance to BA, PT and DMPT was greatly decreased (1/1,000 or lower) in the Bar<sup>-</sup> mutant as compared with the parent. BA production was not detected (1/100 or lower) in the Bar<sup>-</sup> mutant, while a considerable amount of DMPT was accumulated (Table 1). Other BA intermediates (DMBA, PT, AcDMPT, AcPT, AcDMBA and AcBA) were not detected at all. PAT activity and 22 kd protein<sup>5)</sup> corresponding to PAT were lost completely in the Bar<sup>-</sup> mutant. Fig. 4 shows that the Bar<sup>-</sup> mutant can convert AcDMPT to BA.

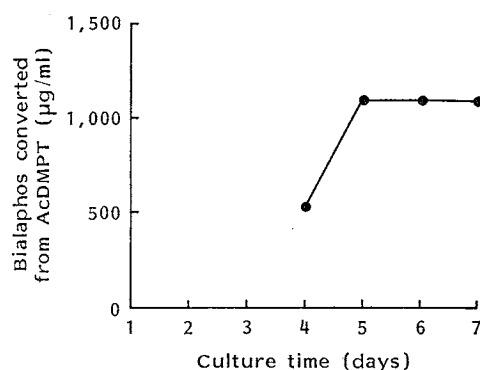
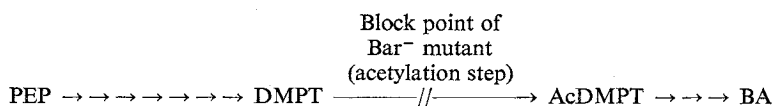
Fig. 4. Conversion of *N*-acetyldemethylphosphinothricin (AcDMPT) to bialaphos by Bar<sup>-</sup> mutant.

Table 2. Enzyme activity and intracellular protein.

Strains	Enzyme activities (u/mg protein)		22 kd protein
	PAT	GS	
Parent	0.2	10	+
Bar <sup>-</sup>	ND	7	-
Bar <sup>-</sup> /pBG3 ( <i>bar</i> plasmid)	1.0	8	+
Bar <sup>-</sup> /pGSC1 ( <i>glnA</i> plasmid)	ND	125	-

PAT: Phosphinothricin acetyltransferase, GS: glutamine synthetase, ND: not detected.

Fig. 5. The biosynthesis of bialaphos.



PEP: Phosphoenolpyruvic acid, DMPT: demethylphosphinothricin, AcDMPT: *N*-acetyldemethylphosphinothricin. See more details in refs 13~15 and 23~26.

#### Restoration of Parental Phenotypes in Bar<sup>-</sup> Mutant by Normal *bar* Gene and *glnA* Gene

In order to confirm the role of *bar* gene, plasmid containing normal *bar* gene was introduced into the Bar<sup>-</sup> mutant and examined for restoration of parental phenotypes. As shown in Tables 1 and 2, parental phenotypes such as BA resistance, BA production and PAT activity were completely restored by the introduction of the normal *bar* gene (pBG3) into the Bar<sup>-</sup> mutant. Since it might be possible that restoration of BA production was due to a secondary effect of restoration of BA resistance, it was attempted to restore BA resistance specifically by introducing *glnA* gene. The *glnA* transformant (Bar<sup>-</sup>/pGSC1) exhibited BA resistance but not BA production. DMPT accumulation of Bar<sup>-</sup>/pGSC1 was almost the same as that of Bar<sup>-</sup> mutant. Overproduction of glutamine synthetase was observed in the Bar<sup>-</sup>/pGSC1 (18 times as compared with the Bar<sup>-</sup> mutant) (Table 2).

#### Discussion

As described so far, construction of Bar<sup>-</sup> mutant by gene replacement technique resulted in the simultaneous loss of BA resistance and BA production and increased production of DMPT without a change in conversion ability of AcDMPT to BA. Introduction of the normal *bar* gene restored all the phenotypes that the Bar<sup>-</sup> mutant lost. On the other hand, introduction of *glnA* gene into Bar<sup>-</sup> mutant restored BA resistance but not BA production. It was thus concluded that the *bar* gene plays a role of both self-defence and the acetylation step of BA biosynthesis in BA producing *S. hygrosopicus*. There has been many indirect evidences that the self-resistance determinant plays a role in a certain step of antibiotic biosynthesis. This was supported by facts that antibiotic production genes are physically linked to the gene coding for antibiotic resistance<sup>20-22</sup>.

Our specific approach in this study is the first case that provided the direct and conclusive evidence for the role of self-resistance determinant. This approach can be generally applicable and useful to elucidate the role or function of self resistance determinants in various antibiotic producers.

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